Final report NASA-Ames NCC 2-764
"Fludrocortisone: Role in central regulation of fluid balance"

Period covered: 7/1/93-1/15/96.

We have performed 7 experiments in rats on the effects of systemic treatment with fludorocortisone (9a-FF) given in a variety of doses over a variety of times, in the AM and in the PM. These experiments were designed to determine the best treatment protocol to use in the head-down rat model studies which were performed at NASA-Ames Research Center during the final year. The results of the experiments in non-stressed rats have been, on the whole, disappointing in that we have so far been unable to obtain direct evidence that the 9a-FF acts as a mineralocorticoid. In the ultimate experiments using control and suspended rats, we showed mineralocorticoid and glucocorticoid effects of 9aFF. However, suspension served in our rats as a chronic stressor when they were examined 5 d after the onset of treatment. Below is a list of the experiments that we performed and the results.

Experiment 1. To devise infusion doses of 9a-FF that should be used in intact rats, we used adrenalectomized rats and attempted to modify the injection protocol used by Lim and Funder (J. Clin Invest. 69:1191-1198) to our intended constant infusion protocol. Young male Sprague Dawley rats weighing 150-175 g were adrenalectomized and implanted at that time with Alza miniosmotic pumps which delivered 9a-FF at rates of 0.5, 1.0 and 5 ug/d for 5 days. As in the Lim and Funder experiments, the rats were maintained on saline to drink (0.5% NaCl). The animals lost weight in proportion to the rate of 9a-FF infusion (as seen by Lim and Funder). However, we not only measured endpoints associated with activity of the hypothalamo-pituitary-adrenal (HPA) axis, but also measured plasma electrolytes and osmolality. The experiment was a catastrophe; the rats were scrawny and unwell, HPA axis activity was increased in proportion to the dose of steroid infused, probably because the rats were sick. With hindsight, we recognized that the steroid infused animals should have been taken off saline and allowed to drink water. 9a-FF has a short half life and Lim and Funder probably got away with keeping their rats on saline because they injected the steroid only once a day, whereas we were maintaining a constant elevation in steroid by infusion. Thus, the results of experiment 1 were not revealing.

Experiment 2. In this experiment, intact rats were implanted with osmotic pumps designed to deliver 0, 0.5, 1 and 5 ug 9a-FF/day for 5 days, and the rats were, of course, allowed to drink water. Three rats from each group lived in metabolism cages during the experiment; food and water intakes were measured daily as was urinary Na, K and osmolality. On the morning of d 5, rats were stressed by being placed in restraining tubes and having blood samples collected from the tail at 0 and 15 min; the rats were decapitated at 30 min.

Results: Again we ran into complexities that we had not anticipated. Because 9a-FF has high, and equal, affinity for both the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), we observed potent interactive effects on both systems across the range of doses used. These are difficult, if not impossible, to sort out.

Mineralocorticoid receptor-mediated effects. In terms of effects mediated probably by association of the steroid with MR, we observed a significant overall effect of dose on hematocrit, with significant decrease at the highest dose ($p= 0.023$). This result suggested that at the highest dose at least, the steroid caused expansion of plasma volume as anticipated. Examination of urinary Na^+ excretion showed little effect on days 1,2 and 4 but slightly decreased excretion at all doses of steroid on d 3-4, which could account for the expansion observed on d 5 am. Water intake also tended to increase with increasing doses of 9a-FF, agin compatible with expansion of the ECF volume. However, if these effects were meaningful, we should have observed consistent, dose-related increases in body weight gain over the infusion period and we did not. The 2 lower doses caused slight gains in body weight above that observed in vehicle-infused controls. The group of rats given the high dose, however, gained the same amount of weight as the controls over the 5 d period. We believe, see below, that this is because the group given the high steroid dose exhibited some clear GR-mediated effects which clearly reduced food efficiency and was on the border of typical glucocorticoid-mediated catabolic actions.

Glucocorticoid receptor-mediated effects. Food intake increased slightly but consistently with increasing rates of steroid infusion. Although pituitary ACTH concentration and the plasma ACTH response to stress did not change as a function of steroid infused, adrenal weight was significantly reduced by the steroid in a dose-related fashion ($p=0.009$), with the highest dose causing significantly smaller adrenals. Adrenal corticosterone (B) content tended to decrease in the stressed rats with steroid infusion ($p = 0.108$), however there was no effect of the steroid infusion on thymus weight. This result would be anticipated, since it is likely (although not measured here) that the exogenous infusion of glucocorticoid caused the endogenous system to decrease its activity to the extent possible. It seems likely that only at the highest dose of steroid infused was the endogenous system overwhelmed (as judged by adrenal weight). Plasma glucose concentrations in the 30 min stress samples were uniformly high.

From this experiment we thought the results were encouraging enough that a further experiment using chronic treatment was warranted.

Experiment 3. In this experiment heavier rats were used (final weight ~ 250-270g) and oil, 2, 10, or 20 ug/rat/day 9a-FF was given in the morning by intramuscular injection for 4 d. We hoped with this approach to increase the mineralocorticoid and decrease the glucocorticoid effects of the steroid. Again, 3

rats from each experimental group lived in metabolism cages over the 5 d. The results were disheartening. Presumably because the last steroid injection had been given 28 h previously, there was no effect of the steroid treatment regimen on plasma volume or urinary Na excretion after the volume load. However, there were also no significant effects of the steroid treatment on any of the endpoints examined, although there was a tendency toward reduced adrenal and thymus weights with dose of 9a-FF, and a consistent, but non-significant tendency for increasing steroid dose to inhibit the ACTH response to restraint (Fig. 1). There were no discernable mineralocorticoid effects in the endpoints measured. If anything, urinary Na+ excretion increased with increasing steroid; however that may have been a consequence of the slightly increased food intake that occurred as a function of the steroid dose, since the rats ate normal rat chow that has a high Na content.

We abandoned the chronic treatment approach as unsuitable and adopted a new approach that is more in line with the preventive treatment paradigm now being examined for efficacy in ground based simulations. In the **final sets of studies**, groups of rats were injected with 20 ug 9a-FF 0, 1, or 2 X, each injection separated by 12 h, the last injection given 2 h before testing. Vehicle-injected controls were used and all rats received a total of 2 injections of either vehicle or steroid. The studies were carried out in both the AM and in the PM, with the experiments performed entirely at the NASA-Ames Research Center. All of the previously mentioned endpoints were measured and, 2 h after the final injections of 9aFF or vehicle, rats were injected under ether anesthesia with Evans' Blue for determination of plasma volume after they were killed 10 min later. The animals were either treated as controls or were placed in suspension for 5 d prior to being injected at -14 and -2 h with either 0, 1 or 2 doses of 9aFF.

Results: In encapsulated form, the conclusion from these studies is that suspended rats are chronically stressed. Although we saw the largest effects of 9aFF on body Na whan only one injection was given 2 h before killing the rats, this effect was essentially swamped by the chronic effects of tail suspension when these rats were compared to control. When 2 injections of 20 ug 9aFF were given at 12 h intervals (14 and 2 h prior to death of the animals), endogenous responses to the first injection appear to have swamped the effects of the second injection.

Fig 2. Shows changes in body weight and food efficiency across the 5 days of the study. The increase in body weight was significantly diminished in the suspended rats, both in the AM and PM studies. This is because, although food consumption was only marginally decreased, food efficiency (grams of weight gained/gram of food consumed) was markedly and consistently reduced in the suspended rats.

Fig 3. shows urinary Na output during days 2-4 of suspension. Although there was no difference in Na output in the AM study, there was a significant overall effect of suspension in reducing Na output because in the PM group, there was a highly significant decrease in Na output in the suspended, compared to the control rats. This was true for both the AM and PM groups during the last

24 h of suspension; however, injection of 9aFF 14h prior to killing the rats caused an increase in Na output in the suspension group in the AM and in the control group in the PM. One injection of 9aFF 2 h prior to death caused a significant decrease in plasma protein, suggesting the expected expansion of plasma volume, in all groups of rats treated with the mineralocorticoid; however after 2 injections of 9aFF, 14 and 2 h prior to death, the rats had apparently accomodated the the mineralocorticoid injection and there were no differences from untreated rats in plasma protein.

Fig 4. shows adrenal (top) and thymus (bottom) weights for these rats. There was a significant increase in adrenal weight in the suspended rats compared to controls again, as with body weight gains and caloric efficiency, suggesting that chronic suspension is a chronic stressor. The degree of chronic stress (in terms of putative excessive corticosterone secretion) is mild, since there was not a significant effect of suspension on thymus weight. Markedly elevated corticosterone over 5 days would have decreased thymus weights significantly.

Fig 5. shows plasma renin activity (top) and aldosterone concentrations (bottom) 10 min after the onset of ether stress and injection. These values clearly represent stressed levels of both substances. However, renin responses to ether were greater in the suspended rats than in the controls at both times of day, suggesting that there is facilitation (see below) not only in the adrenocortical system but also in the sympathetic nervous system as a consequence of chronic stress. The fact that 9aFF injections markedly reduced the renin responses in the suspended rats to ether stress suggests that the steroid does act centrally on SNS output to the periphery to inhibit this system when it is excited. By contrast, to the renin responses which are not affected by the ACTH response to acute stress, the aldosterone responses reflect primarily the ACTH secretion that occurred after ether (see Fig 6). Aldosterone responses in the suspended rats were greater than control in the AM but not in the PM. Prior treatment with 9aFF reduced the aldosterone responses in a dose-related fashion at both times of day (except for the small response in the AM control rats).

Fig 6. shows plasma ACTH (top), and adrenal (middle) and plasma corticosterone (B) responses (bottom) 10 min after ether in the AM and PM in control and suspended rats. That suspension is a chronic stressor is unequivocally established by the ACTH results. In prevous and subsequent studies of the effects of chronic stress on responses to acute novel stress (using either streptozotocin-diabetes or cold for 5 d; [Scribner, 1993; Akana, 1994; Akana, 1992; Akana, 1997; Akana, 1996]), we have observed that the previously stressed rats hypersecrete ACTH to novel stress in the AM but not the PM. This is clearly the case with rats that have been suspended for 5 d as well. There was a significantly greater ACTH response to ether in the AM in the suspended rats, compared to controls, and no difference in the PM from controls. Furthermore, as shown previously with corticosterone treatment, inhibition of the ACTH response by 9aFF was highly significant in the AM but far less so in the PM. Adrenal and plasma B, again as in previous studies, show facilitation in the PM, not the AM; just the opposite from ACTH. However, we had no basal samples in these studies, and in previous studies we showed that the effect of facilitation in

the PM was on basal levels of ACTH and B and that the magnitude of the response to stress was the same in previously stressed vs control rats[Akana, 1992; Akana, 1997].

In summary: The experiments show that a single, but not multiple doses of 9aFF does work to expand extracellular fluid volume in rats, as in man. Furthermore, rather than stimulating the sympathetic neurvous system as it was hypothesized it might, it appears that 9aFF may inhibit the response of the SNS to acute stress (based on the the inhibited PRA response to ether stress). Tail suspension of rats as a model for weightlessness in man definitely appears to be a persistant stressor over at least the first 5 d, based on decreased body weight gain, caloric efficiency, enlarged adrenal glands and hyperresponsivity of the HPA axis to a novel stress in the AM. This effect of suspension in rats resembles, we believe, the effects of space flight in man although the stressor in rats is probably not the same as that in man. In reports of urinary cortisol excretion during space flights, on average cortisol excretion doubles (JAP, July 1996). In man, the persistent stressor encountered during space flight may be either the heavy work load, the early alterations in appetite accompanied by nausea or the possible lack of a normal circadian rhythm in cortisol. Whatever the proximate cause of the increased cortisol excretion (and probably adrenal secretion) during space-flight, the elevated cortisol concentrations would be expected to reinforce the negative nitrogen balance that is ascribed to muscle atrophy and the immune system dysfunction that have been observed in space. It is interesting that rats in space show no signs of excessive corticosterone secretion (JAP, 1996). Clearly in the best ground-based simulation, tail tweaking involved with suspension is a stressor - as shown by Alonso et al. (AJP, 1994) in both suspended and non-suspended but tail-treated rats compared to untreated controls. These animals may still serve as useful models for man in space, simply because they exhibit characteristics of elevated ACTH and corticosterone secretion similar to those observed in man in space.

Submitted 1/16/98.

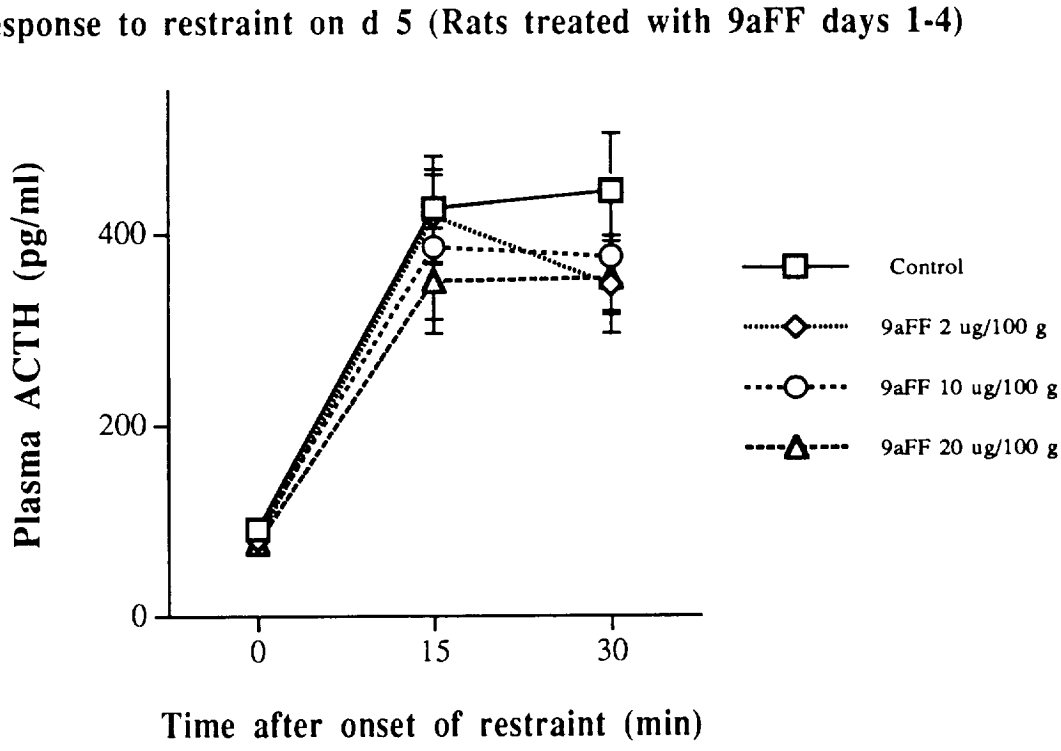
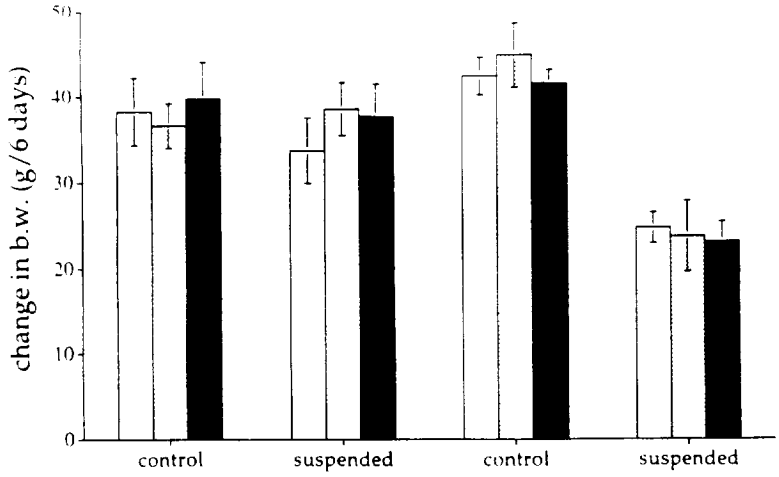


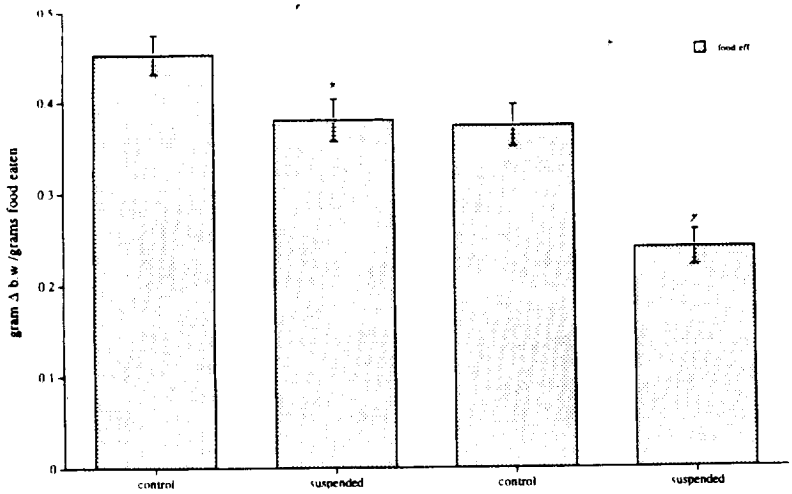
Figure 1. Final Report NCC2-764

Change in body weight

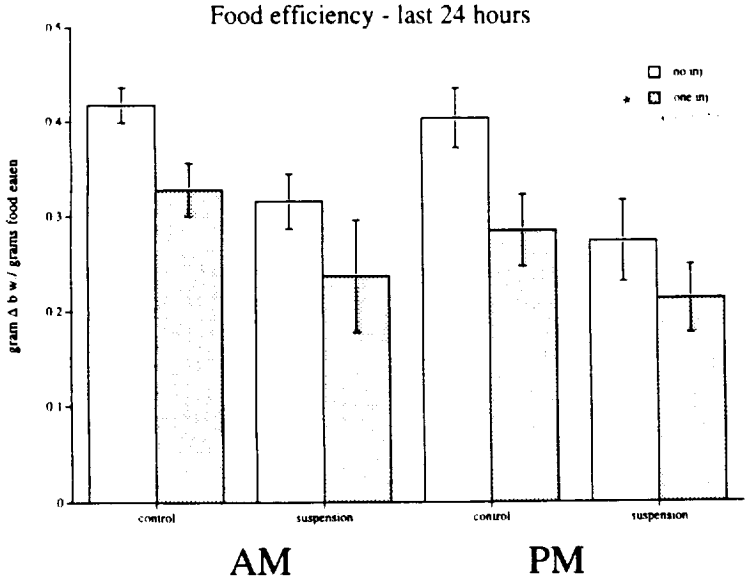


control
20 μg 9αFF -2h
20 μg 9αFF -2,-14h

Food efficiency days 2-4 of suspension



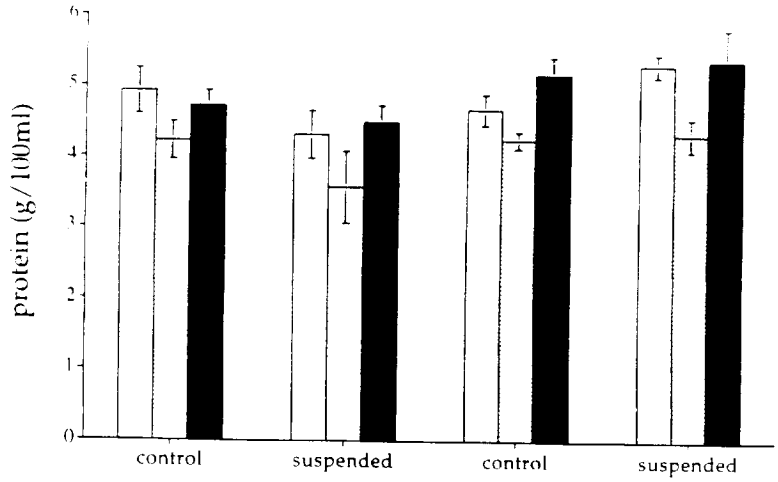
Food efficiency - last 24 hours

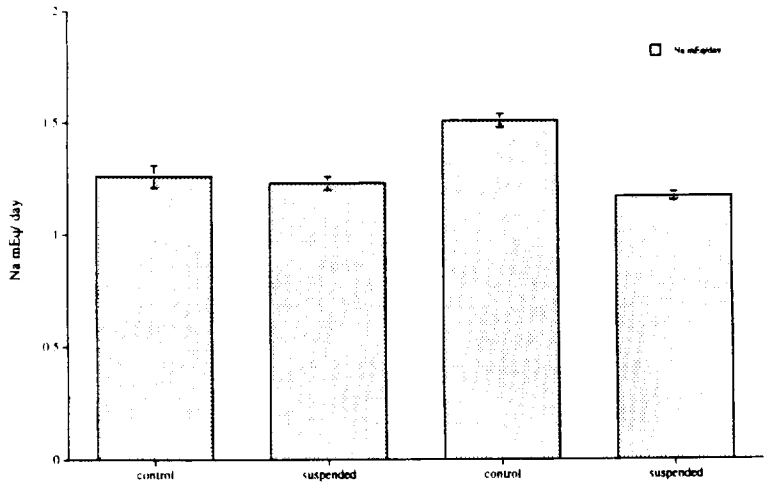


control
9αFF-14h

Fig. 2 Final Report NCC 2-764

Control
9αFF 20μg -2h
9αFF 20μg -2,-14h





control
9αFF -14h

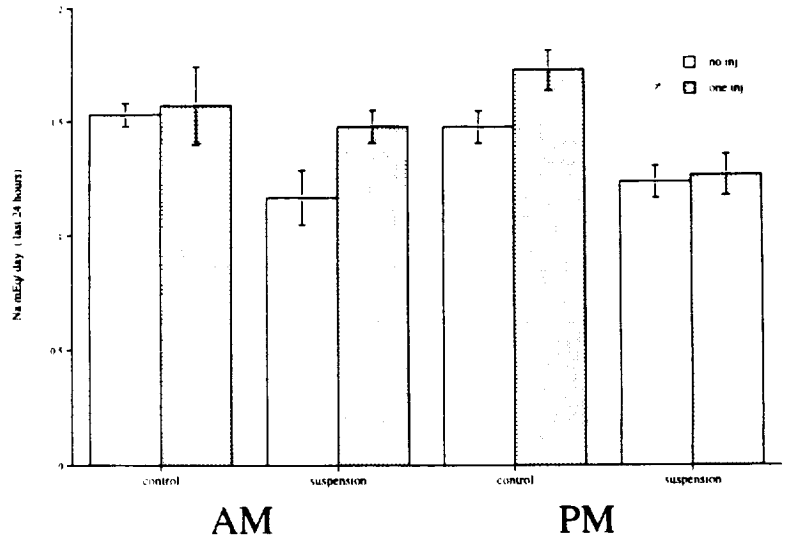
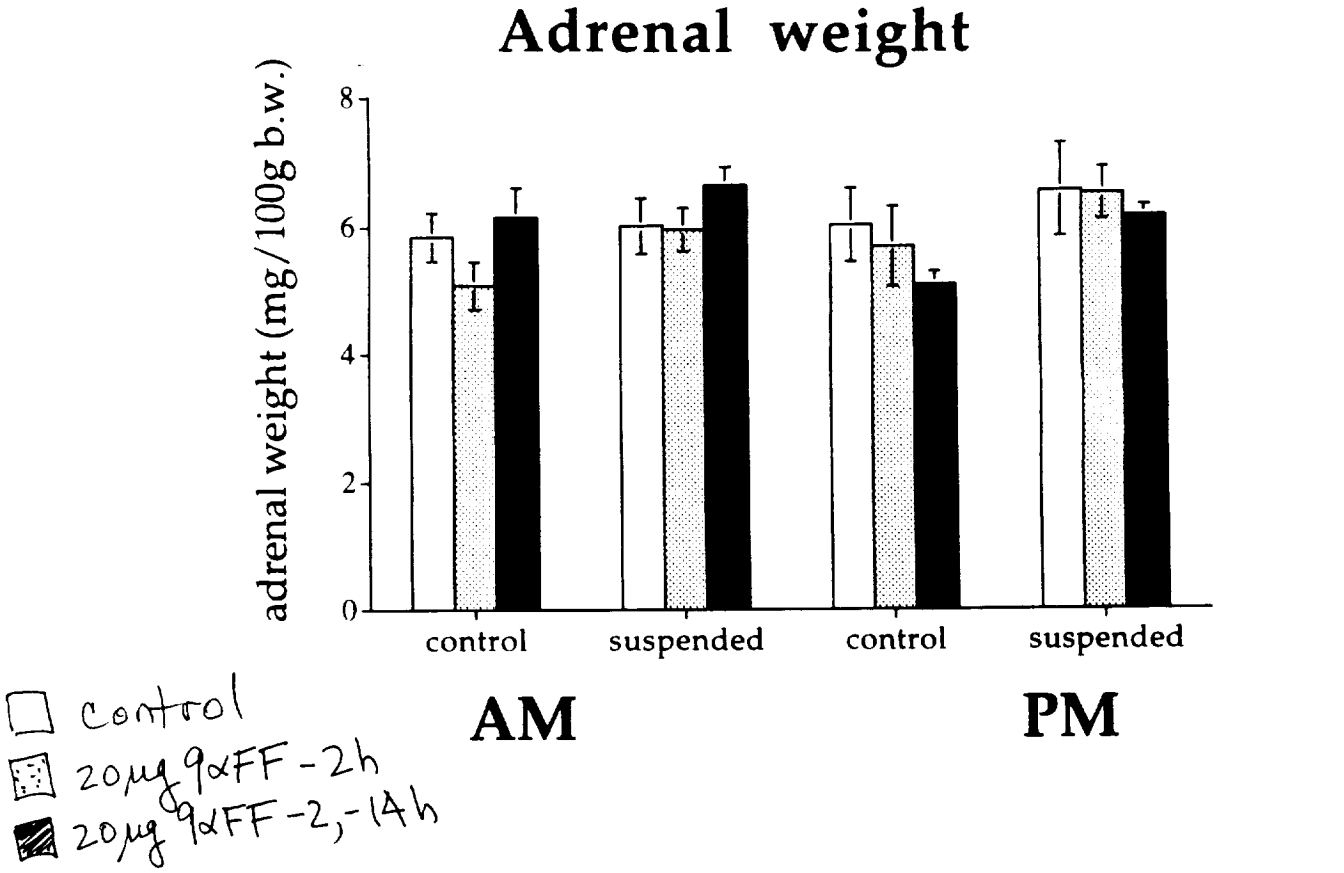


Fig. 3 Final Report NCC 2-764



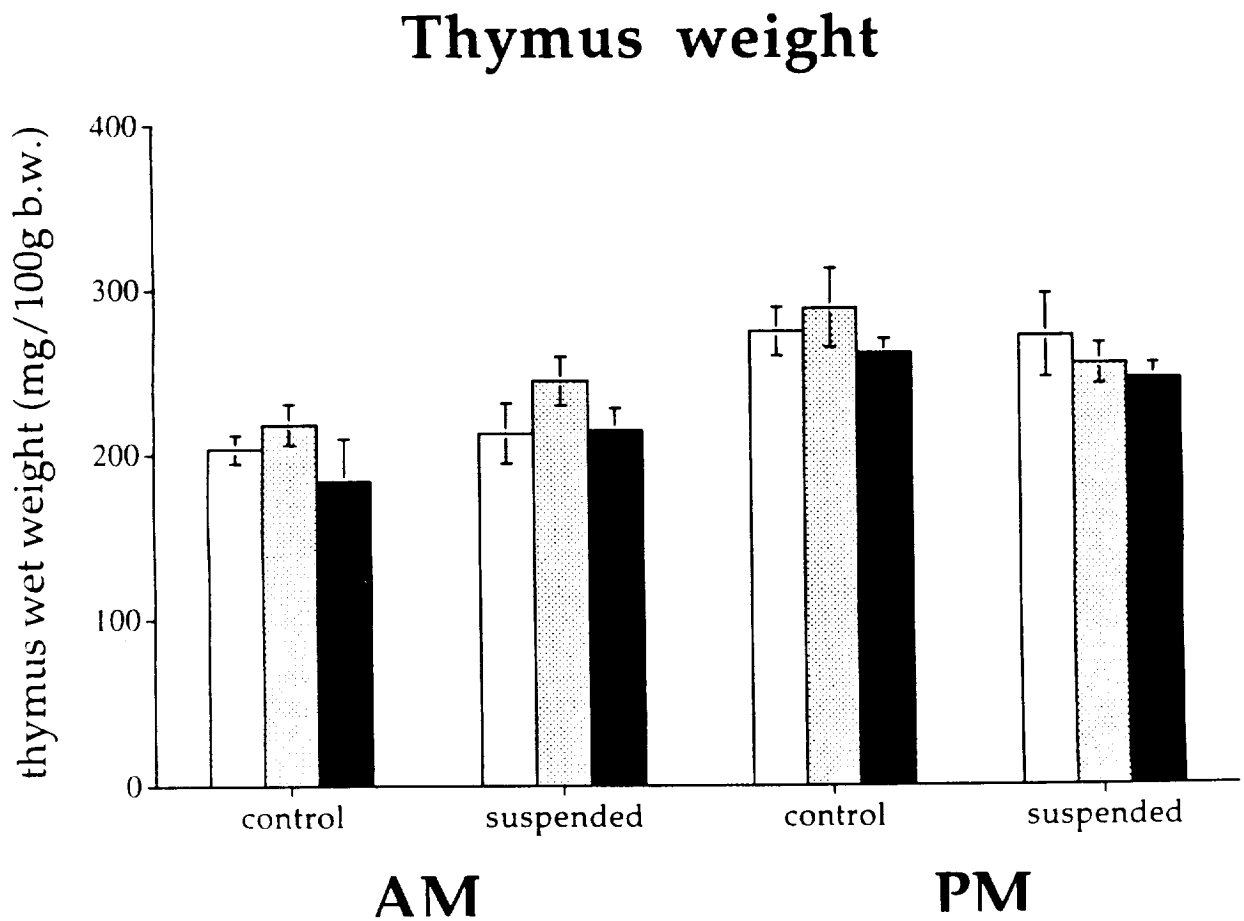


Fig 4. Final Report NCC2-764

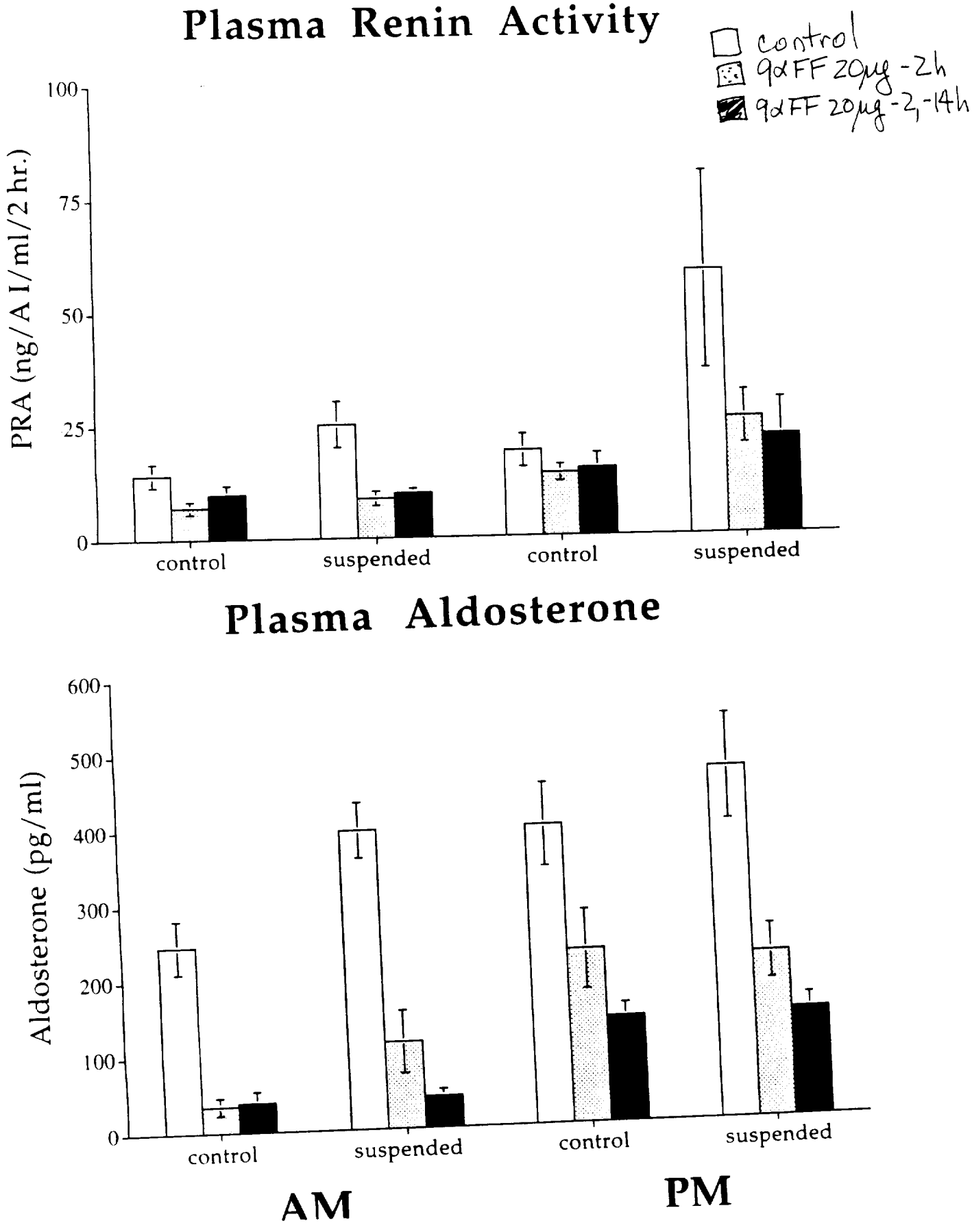


Fig 5. Final Report NCC2-764

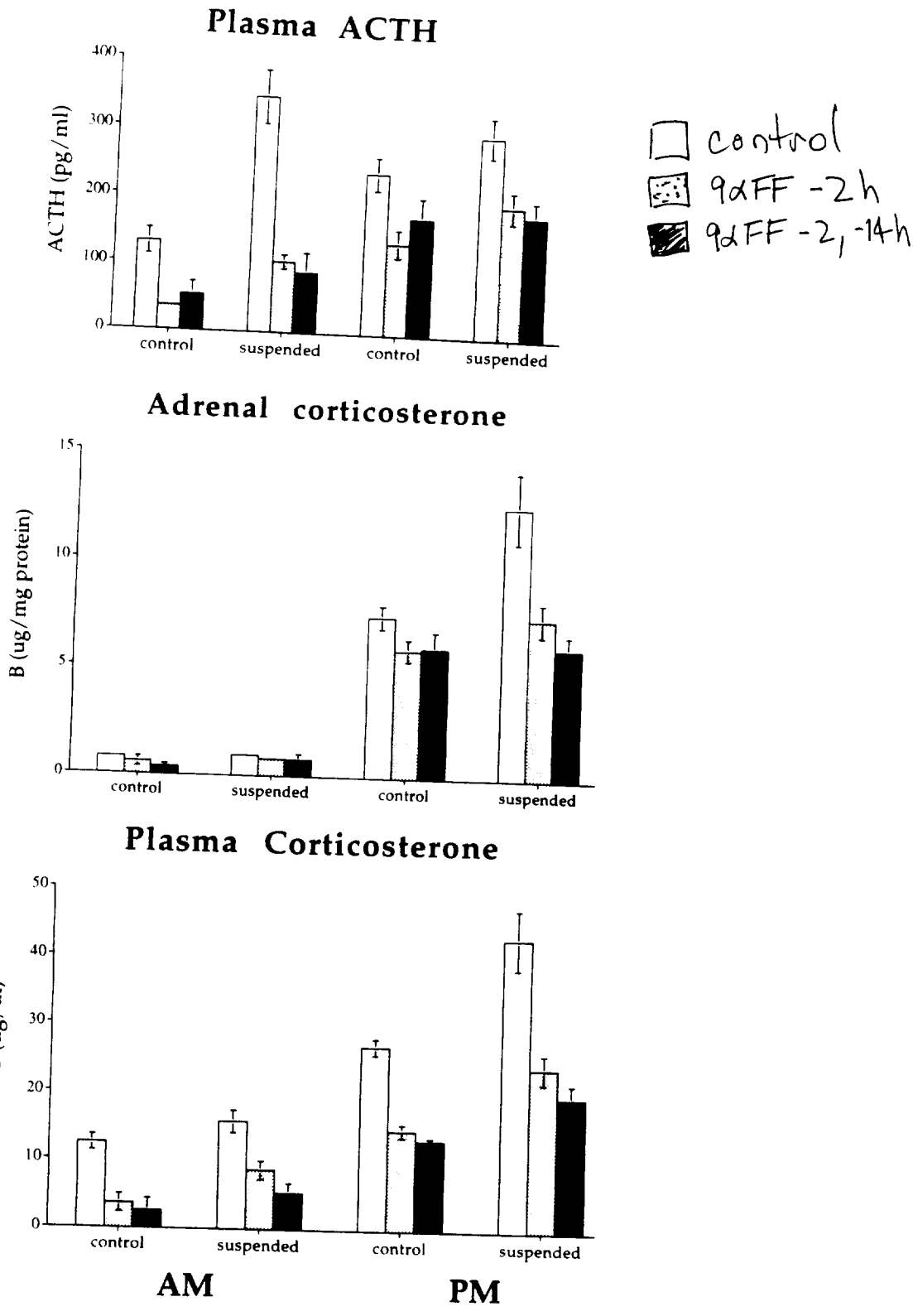


Fig. 6. Final Report NCC2-764

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Reply to Attn of: JAC: 241-1

Ms. Melanie Schafmeister
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San Francisco, CA 94118-0962

December 30, 1997

Subject: Grant No. NCC 2-764 , P.I. Mary F. Dallman .

The subject Agreement has ended on Dec. 31, 1995. Your institution was found to be non-compliant based on non-receipt of the following reports: Final Inventions Report and Final Technical Report. The agreement was administratively closed by the Grant Office.

Failure of the recipient to provide a required grant report can result in the Agency and the public being denied information about the grant activities, NASA officials having less information for making decisions, grant close-out being delayed, and confidence being undermined as to whether the recipient will meet the requirements under other grants.

NASA may in addition to imposing any of the Special Conditions outlined in section **(1260.114)** of the NASA Grant and Cooperative Agreement Handbook, elect to use the Enforcement Clause section **(1260.162)** as remedies for non-compliance. This is because NASA Grants provide for advance payments and recipients are paid before final reports are due. At this point it is too late to withhold payment on the existing grant. Therefore withholding special condition **(1260.56)** may be used when awarding a new grant or modifying an existing grant with non-compliant organizations.

Your institution will be added to our list of non-compliant organizations and appropriate action will be determined on a case-by-case basis.

If you have questions feel free to contact the undersigned at: (650) 604-2074, or Venoncia A. Braxton at: (650) 604-5811.

for Beatrice Morales
Grant Officer

cc: 203-18/ J. Timmons, Accounts Payable